A Novel RING Finger Protein Interacts with the Cytoplasmic Domain of CD40*

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Running Title: CD40 binding protein

Summary

CD40 is a member of the TNF receptor family and, like other members, it appears to possess no intrinsic signaling capacity (e.g. kinase activity), suggesting that signal transduction is likely mediated by associating molecules. To identify such molecules, we have utilized the yeast two hybrid system to clone cDNAs encoding proteins that bind the CD40 cytoplasmic domain. One such interacting protein, designated CD40 binding protein, has a N-terminal RING finger motif that is found in a number of DNA binding proteins, including the V(D)J recombination activating gene RAG 1. In addition, it contains a prominent C-terminal coiled-coil segment that may allow homo or hetero-oligomerization. The C-terminus also possesses substantial homology to the TRAF[1] domain that is found in two proteins (TRAF1 and TRAF2) that associate with the cytoplasmic domain of the related 75kD tumor necrosis factor receptor. This is the first identification of a molecule that interacts with CD40 and whose sequence suggests a potential role in signaling.

CD40 antigen is a cell surface transmembrane 45 kD glycoprotein receptor expressed on B-lymphocytes (1) and its ligand, CD40L, is expressed on activated T-helper cells (2). CD40 activation is critical for B-cell proliferation (3), immunoglobulin class switching and rescue of germinal center B-cells from apoptosis following somatic mutation (4,5). Mutations in CD40L result in an immunodeficiency (X-linked hyper-IgM syndrome) characterized by IgM producing B-cells that do not form germinal centers in response to foreign antigens (6,7,8).

CD40 antigen has a short cytoplasmic tail (65 amino acid residues) and mutagenesis studies suggest that Thr_{234} in the cytoplasmic domain is essential for signal transduction (9). Since traditional methods, including co-immunoprecipitation and chemical cross-linking, have failed to identify molecules associating with the cytoplasmic domain, we used the yeast two hybrid system as an alternate means to identify interacting molecules (10).

Materials and Methods

Yeast 2-hybrid Screen

A hybrid gene encoding the GAL4 DNA-binding domain (amino acids 1-147), HA epitope tag and CD40 cytoplasmic region (amino acids 216-279) was constructed in the yeast bait vector pAS1CYH2 (11). This construct was designated GAL4CD40 and expression of the fusion protein was confirmed by anti-HA immunoblotting (not shown). This bait plasmid was cotransformed with a human B-cell cDNA expression library (prey) fused to the activation domain of GAL 4 in the pACT plasmid (11). Interaction between bait and prey encoded genes in the Y190 yeast strain reconstitutes GAL 4 as an active transcriptional complex, allowing growth in the absence of histidine and activation of the β-galactosidase reporter gene. Thirty-six of the 10^6 transformants screened grew in the absence of histidine and had detectable β-galactosidase staining within 10 minutes of incubation with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside. Plasmids recovered from the original yeast strains were used in a cotransformation assay with GAL4CD40 or control heterologous baits. 12 plasmids encoded proteins that interacted with native CD40 but not with the control heterologous baits. DNA sequencing revealed 9 of the 12 to encode the same protein designated CD40 binding protein (CD40bp). Cotransformation assays were repeated in the yeast Y190 strain (11) where CD40bp fused to the activation domain of GAL 4 was cotransformed with native CD40 (pCD40T) or the indicated heterologous baits expressed as fusions with the DNA-binding domain of GAL4. These included mutant CD40 where Thr_{234} was changed to an alanine (pCD40A), the cytoplasmic domain of the p55 TNF receptor (pTNFR amino acids 206-426), FAS receptor cytoplasmic domain (pFAS amino acids 178-319), truncated p55 TNF receptor cytoplasmic domain missing 20 C-terminal residues (ΔTNFR amino acids 206-406), the helix-loop-helix motif of E12 (amino acids 508-564) (12) and the yeast ser-thr kinase SNF1 (13). Colonies from each transformation were patched onto a selective plate and a β-galactosidase assay performed on yeast transferred to nitrocellulose filters and permeabilized in liquid nitrogen (11).

GST Fusion Protein Expression and *in-vitro* Binding Assay

Native (CD40T) and mutant (CD40A) CD40 sequences used in the construction of the yeast bait vectors were excised and subcloned into the GST fusion protein vector pGSTag (14) and transformed into the *E. coli* strain BL21(DE3) pLysS (15). GST and GST fusion proteins were prepared using published procedures (15) and the recombinant proteins were immobilized onto glutathione-agarose beads at a concentration of 8 mg/ml (11).

Labeled CD40bp was prepared by *in vitro* transcription/translation using the TNT T7 coupled reticulocyte lysate system from Promega according to the manufacturer's instructions. Briefly, a 2.2 kb cDNA encoding CD40bp was excised from the yeast prey vector (pACT) using Xho 1 and subcloned into the pBluescript II plasmid (Stratagene) which had a flanking T7 promoter allowing generation of sense strand transcript. The luciferase construct was provided by the vendor and could similarly be transcribed by T7 polymerase.

Following translation, 5 µl of total ^{35}S-labeled reticulocyte lysate was either subjected to SDS-PAGE and fluorography or diluted into 1 ml GST binding buffer (10 mM Tris, pH 7.6, 50 mM NaCL, 5 mM EDTA, 1% Triton X-100, 0.1% BSA, 1 mM PMSF) and incubated with either 40 µl of a 50% slurry of GST, GSTCD40T or GSTCD40A agarose beads for 2 hours at 4°C, following which the beads were pelleted by pulse centrifugation in a microfuge, washed three times in GST buffer (without BSA), boiled in SDS-sample buffer and resolved on a 10% SDS-acrylamide gel. Bound proteins were visualized following autoradiography at -80°C for 1 hour.

Construction of CD 40 and CD40bp expression vectors

Full length CD40 coding sequence was obtained by PCR from a human B-cell library (11) and confirmed by sequencing. The primers used were: CGGGGTACCGCCACCATGGTTCGTCTGCCTCTGCAG for the upstream primer and TTTGTCGACTCACTGTCTCTCCTGCAC for the downstream primer. The upstream primer had a built-in Kpn 1 site and the downstream primer a Sal 1 site (underlined) to facilitate cloning into the eukaryotic expression vector pcDNA3 (Invitrogen). Mutant CD40 (pCD40A) was made by

site-directed mutagenesis using a two step PCR protocol (16) and employing two additional oligonucleotides:
GCTCCAGTGCAGGAAGCTTTACATGGATGC and
GCATCCATGTAAAGCTTCCTGCACTGGAGC (altered bases are underlined). The Thr_{234}—›Ala_{234} mutation in pCD40A was confirmed by sequence analysis.

To construct pHATagCD40bp, CD40bp was excised from the yeast vector pACT by Xho 1 digestion and subcloned into pcDNA3 in which an HA-epitope tag (YPYDVPDYA) had previously been placed downstream of the cytomegalovirus promoter/enhancer. The orientation of CD40bp and the junctional sequence between the HA-tag and CD40bp was confirmed by sequence analysis.

Transfection, metabolic cell labelling and immunoprecipitation analysis

These were performed essentially as described previously (17). For re-immunoprecipitation analysis, the initial immune complex was dissociated by boiling in PBS + 1% SDS, diluted tenfold in PBS containing 1% triton X-100 and 1% deoxycholate and subjected to a second round of immunoprecipitation analysis.

Transcript analysis

mRNA analysis by Northern blotting and RT-PCR was performed as described previously (17). For the Northern blot, 7μg of poly A^+RNA from SKW6.4 cells was hybridized to a ^{32}P-labelled CD40bp encoding Xhol fragment.

For RT-PCR, CD40bp specific 18-mer oligonucleotide primers were used. The downstream primer (AGAGGAGTTGCCTTCTGC) was used initially for the reverse transcription reaction and later for PCR in conjunction with an upstream primer (GGCATGACCAGATGCTGA) to give an expected size product of ~600bp on agarose gel electrophoresis.

DNA sequencing and database searching

Double stranded plasmid template was sequenced on both strands as described previously (17) using modified T7 DNA polymerase. Initial database homology searching (18) revealed hundreds of matches to myosins and other alpha-helical, coiled-coil proteins (not shown). To further characterize portions of the CD40bp that might have coiled-coil potential, we used the COILS 2 program of Lupas et. al. (19) that has been recently updated and is available from lupas@vms.biochem.mpg.de.

Results and Discussion

Nine independent clones were found to encode the same protein, designated CD40 binding protein (CD40bp), that specifically bound the native form of CD40 (pCD40T) in the yeast Y190 strain (Fig. 1). To assess whether the interaction of CD40bp was specific to native CD40, a mutant CD40 bait was created in which Thr_{234} was converted to Ala (pCD40A), an alteration known to disable CD40 signaling (9). In addition, other heterologous baits, including the cytoplasmic domains of the related TNF and FAS receptors, were tested in a cotransformation assay. As shown in Figure 1, CD40bp interacted with native CD40 only, but not with mutant CD40 or the other heterologous baits, suggesting that the CD40-CD40bp interaction was specific as measured by the yeast cotransformation assay.

To independently confirm the CD40-CD40bp interaction, the identical cytoplasmic domain regions of CD40 and mutant CD40 used in the yeast two hybrid system were expressed as GST fusion proteins, immobilized to glutathione agarose beads and used to precipitate radiolabeled *in vitro* translated CD40bp. Figure 2 shows *in vitro* translated CD40bp migrating with an apparent molecular weight of 64 kd, which closely approximates the predicted molecular weight. CD40bp could be effectively precipitated by native CD40 (GSTCD40T), but not by GST alone or, more significicantly, by mutant CD40 (GSTCD40A). Further, none of the GST proteins precipitated luciferase, a control for nonspecific binding. These studies further prove the specificity of the CD40-CD40bp interaction and implicate Thr_{234} in the CD40 cytoplasmic domain as being fundamentally important in both signaling and CD40bp binding.

To demonstrate the interaction *in vivo*, 293T cells, a human epitheloid cell line (that is CD40 negative), were cotransfected with a HA epitope-tagged CD40bp expression construct and either vector alone, mutant CD40 (CD40A) or native CD40 (CD40T) expression constructs. Following metabolic labeling with ^{35}S-methionine and cysteine, cell lysates were subjected to an immunoprecipitation analysis with an anti-CD40 monoclonal antibody (Figure 3A). No labeled protein was immunoprecipitated in vector transfected cells while, as expected, CD40 was immunoprecipitated in both

CD40A and CD40T transfectants. However, only in cells transfected with native CD40 (CD40T) was there a coprecipitating protein whose molecular weight corresponded to CD40bp. To confirm the identity of the precipitating proteins in the CD40T transfected cells, the immune complex was dissociated and subjected to a second round of immunoprecipitation, as shown in Panel B, with either control anti-thrombospondin (α-TSP) antibody, anti-CD40 monoclonal antibody or anti-HA epitope tag antibody (to identify HA-tagged CD40bp). While no labeled protein was precipitated by the control antibody, the αCD40 and αHA tag antibodies confirmed the presence of CD40 and CD40bp in the original immune complex.

To conclusively show that CD40bp interacted with native CD40 in B-cells, the EBV negative, CD40 positive human B-cell line, BJAB, was transiently transfected with the epitope-tagged CD40bp expression construct, metabolically labeled and endogenous CD40 immunoprecipitated with an anti-CD40 monoclonal antibody (Figure 3, Panel C). Autoradiographic analysis of the precipitated proteins following SDS-PAGE revealed, as expected, the presence of CD40 receptor but also that of two associated proteins, one which migrated just larger than CD40bp (Δ) and a fainter band that migrated at the expected molecular weight for CD40bp (*). To confirm that this was indeed CD40bp, the immune complex was dissociated and subjected to a second round of immunoprecipitation with either anti-HA epitope tag antibody or isotype matched control antibody. CD40bp (corresponding to the asterisked band) was clearly immunoprecipitated by the anti-HA antibody and not by control antibody. This confirmed the presence of CD40bp in the original anti-CD40 immune complex and indicated that this interaction was capable of occurring in B-cells. A cautionary note, however, is that the stoichiometry of binding cannot be assessed given the transient nature of the transfection, i.e., only a fraction of cells expressing CD40 were also expressing CD40bp. Expression of CD40bp transcript in B-cell lines was confirmed by Northern blot and RT-PCR analysis (Figure 3, Panels D and E).

The deduced sequence of the 2350 base pair CD40bp cDNA revealed an open reading frame that began with an initiator methionine conforming to Kozak's consensus (20) and that ended 567 residues later at an Opal codon. Given the presence of the open reading frame and the size of the CD40bp

transcript (~2.5kb; Figure 3, Panel D), it is likely that Panel A of Figure 4 represents the full length coding sequence. Homology searching and use of the COILS algorithm (19) revealed a discrete coiled-coil domain spanning residues 266-366 and flanked by regions without coiled-coil potential (Panel D). Residues 266-366 of CD40bp were then "masked" (18) and the database searches repeated. In this case there were 12 statistically significant ($p<0.05$) matches, all to proteins known to contain the "RING finger" DNA-binding motif (21,22,23). Six of the 12 matches (including the most significant match) were to V(D)J recombination activating proteins (RAG1) from various species (24).

Importantly, one of the matches was to the N-terminal RING finger sequence motif of TRAF2, which together with TRAF1, binds to the cytoplasmic domain of the 75kD TNF-receptor as a heterodimeric complex in which TRAF2 contacts the receptor directly (25). It is tempting to speculate that a similar scenario exists for CD40 with CD40bp directly associating with the cytoplasmic domain. The remaining matches included the human RING 1 gene product itself (23), the 52 kD ribonucleoprotein autoantigen in Sjogren's syndrome (26), the *Neurospora* uvs-2 gene product thought to be involved in DNA repair (27), and a developmentally regulated *Dictyostelium* gene (DG17) of unknown function (28). The region between the RING finger and coiled-coil domains contains 17 cysteines and 10 histidines out of a total of 168 residues. These Cys/His residues are arranged in patterns resembling the "B box" motifs observed in some other RING finger proteins (21,22). Neither the RING finger or the coiled-coil segment, a motif known to mediate homo and/or hetero-oligomerization (19) appears necessary for binding to CD40 since one class of interacting CD40bp cDNA's identified in the 2-hybrid screen encoded only the C-terminal half of CD40bp (beginning at Phe_{297} which deletes the RING finger and truncates the coiled-coil segment). Rather, it appears likely that the C-terminal portion mediates CD40 binding.

This is supported by the finding that a similarly truncated TRAF2 protein (missing the RING finger domain) could still associate with the 75kD TNF-receptor (25). In keeping with a common function for the C-termini of these proteins is the remarkable sequence similarity that exists between the C-terminal half of CD40bp and the TRAF domains of TRAF1 and TRAF2

(Figure 4E). Except for the RING finger domain in TRAF2, the three molecules are fairly distinct at their N-terminal halves. Taken together, these studies suggest the existence of a new family of proteins that associate with the cytoplasmic faces of the TNF-receptor family and have in common the TRAF domain. Finally, given that the coiled-coil motif lies within the TRAF homology domain in TRAF1 and TRAF2, it will be important to determine if CD40bp can heterodimerize with these proteins.

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Footnotes

[1]The abbreviations used are: TRAF, TNF receptor-associated factor; TNF, tumor necrosis factor; SDS, sodium dodecyl sulphate; bp, base pair; PCR, polymerase chain reaction; GST, glutathione S-transferase.
[2]GenBank accession number UXXXXX.

Figure Legends

Figure 1. CD40bp interactions with hybrid proteins. Yeast transformants harboring CD40bp fused to the activation domain of GAL 4 and the indicated expression plasmids encoding proteins fused to the DNA-binding domain of GAL 4 were assayed in duplicate for β-galactosidase activity.

Figure 2. Interaction of *in vitro* translated CD40bp with GST fusion proteins. ^{35}S-methionine labeled CD40bp or luciferase protein as control was incubated with either GST alone, GSTCD40T (native CD40 cytoplasmic domain) or GSTCD40A (mutant CD40 cytoplasmic domain Thr_{234}—›Ala). Following incubation and washing, the GST beads were boiled in SDS-sample buffer, resolved on a 10% acrylamide gel and bound protein visualized by autoradiography. The left panel shows the signal from 5 µl of labeled translated protein prior to incubation with GST beads.

Figure 3. Association of CD40 and CD40bp *in vivo* in transfected 293T and BJAB cells. Panel A, 293T cells were cotransfected with HA epitope-tagged CD40bp and with either vector, mutant CD40 (CD40A) or native CD40 (CD40T) expression constructs, metabolically labeled with ^{35}S-methionine and cysteine and cell lysates analyzed by immunoprecipitation with an anti-CD40 monoclonal antibody. **Panel B,** immune complexes from the native CD40T transfected cells were dissociated and re-immunoprecipitated with either control antibody (α-TSP, anti-CD40 (αCD40) or anti-HA tag (αHA) which should recognize HA-tagged CD40bp. **Panel C,** α-CD40 immune complexes from transfected BJAB cells were either analyzed intact (α-CD40) or dissociated and reimmunoprecipitated with an anti-HA tag antibody (α-CD40/α-HA) or isotype matched control antibody (α-CD40/Control Ig). Five fold more cell lysate was used for the double immunoprecipitations. **Panel D,** Northern blot analysis for CD40bp transcript expression in the SKW6.4 B-cell line. **Panel E,** Survey of CD40bp transcript expression by RT-PCR. RNA from the indicated CD40 positive B cell lines (B) and CD40 negative cell lines (T: T cell line; E: Epithelial cell line) was subjected to RT-PCR using CD40bp specific oligonucleotide primers.

Figure 4. Sequence and analysis of CD40bp. The sequence of CD40bp (GenBank accession UXXXXX)[2] is shown in panel **A**. The first underlined segment is the RING finger domain; Cys/His residues that are invariant with respect to other proteins (panel **B**) are indicated in bold. The second underlined region represents the coiled-coil domain (panel **D**). The Cys/His residues between the RING finger and coiled-coil domains are marked by asterisks. Features of the CD40bp sequence are summarized schematically in panel **C**. Homology within the C-terminal TRAF domains of the indicated proteins is shown in panel **E**.

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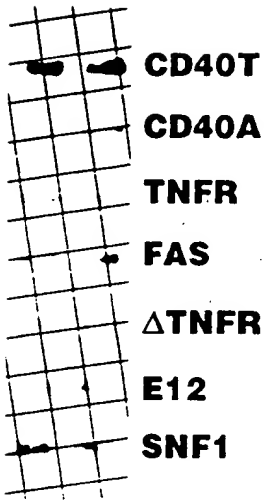
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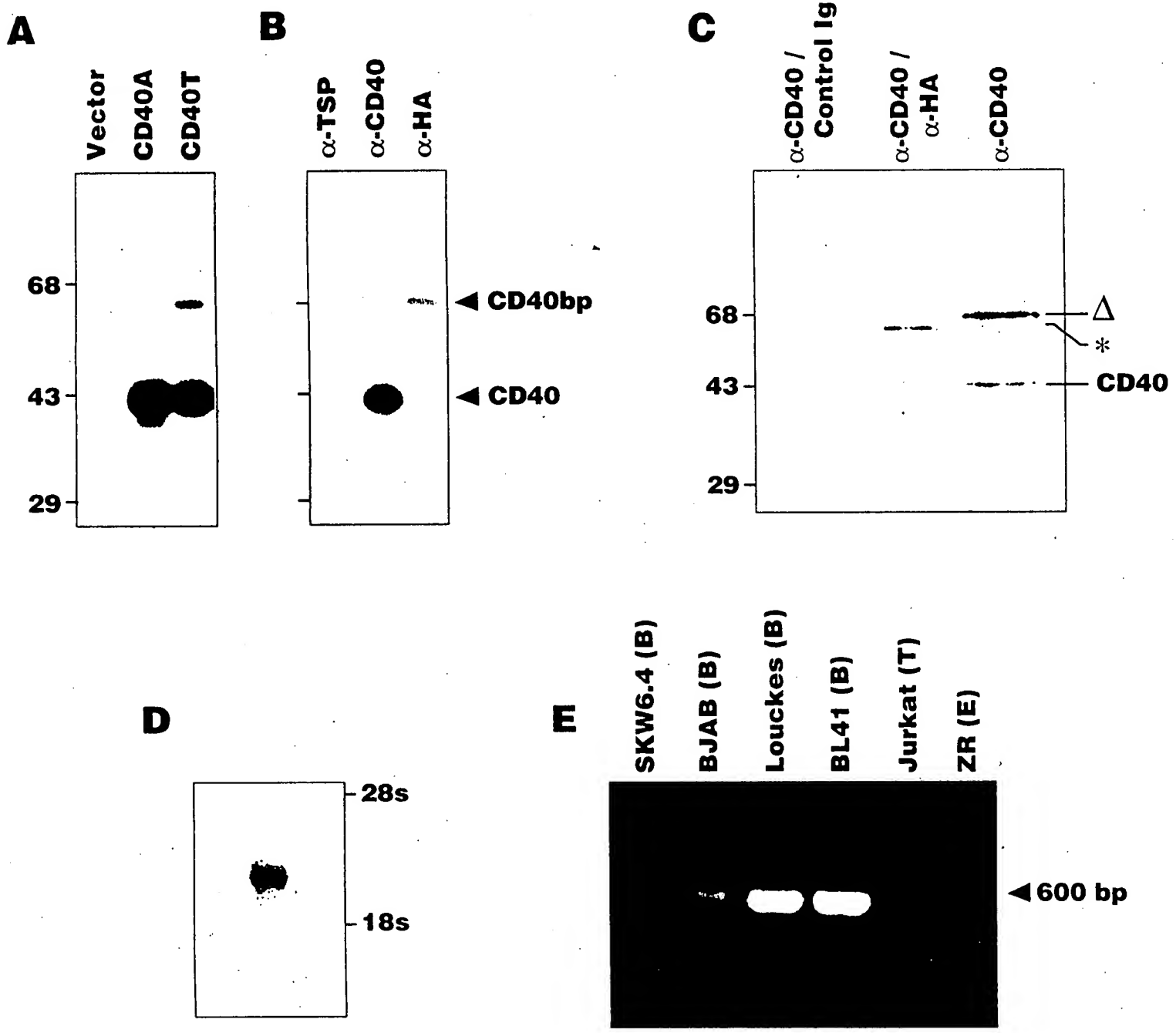
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α-CD40 / α-HA
α-CD40
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43
29
Δ
*
CD40
D
28s
18s
E
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Louckes (B)
BL41 (B)
Jurkat (T)
ZR (E)
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B

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289 KSISCQICEHILADPVET-NC--KHVFCRVCILRCLKVMGSYCPSCR-YPCFP  337  RAG1
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12  EEVTCPICLDPFVEPVSI-EC--GHSFCQECISQVGKGGGSVCPVCR-QRFLL  60   52kd RNP
30  QAFRCHVCKDFYDSPMLT-SC--NHTFCSLCIRRCLSV-DSKCPLCR-ATDQE  77   UVS-2
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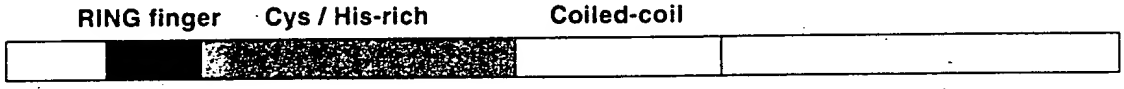

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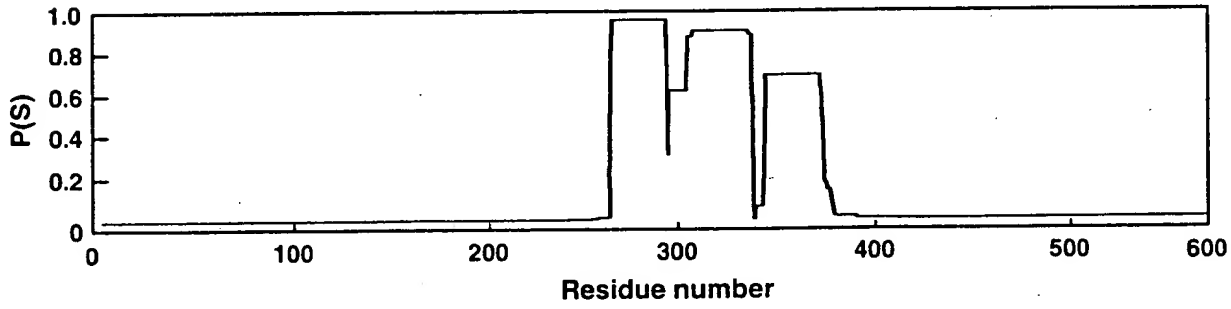

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394 YVKDDTMFLKCIVD-TSA                       TRAF1
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